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Short communication

c-Fos expression in NMDA receptor-contained neurons in spinal cord in a rat model of inflammation: a double immunocytochemical study

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Abstract

Double-labeling techniques were used to demonstrate the nociceptive activation of NMDA receptor-contained neurons in spinal dorsal horn by using c-Fos immunoreactivity as an indicator of this activation in a rat model of inflammation. About 25% and 55% of the c-Fos-immunoreactive neurons that were found in laminae I–II and lamina V showed NMDA receptor immunoreactivity, while about 4% and 11% of NMDA-receptor immunoreactive neurons in these two regions showed c-Fos immunoreactivity, respectively. The implication of the results was discussed.

Keywords: *N*-methyl-D-aspartate receptor; Immediate early gene; Immunocytochemistry; Pain; Spinal cord; Rat

Several lines of evidence suggest that glutamate plays an important role in the spinal transmission and modulation of nociceptive inputs, mediated by several members of a large family of glutamate receptors [1,7]. Glutamate receptors have been classified into G-protein coupled metabotropic receptors and ligand gated ionotropic channels [19]; the latter have been further distinguished according to their preferred agonists as NMDA (*N*-methyl-D-aspartic acid), AMPA (α-amino-3-hydroxyl-5-methyl-isoxazol-4-propionic acid), and kainate subtypes. The cloning of cDNA encoding rat NMDA receptor subunits has revealed five subfamilies of subunits designated as NMDA NR1 and NR2A-D [12]. The NMDA NR1 subunit, yielding eight possible splice variants by three alternative splicing events [18,25], is an essential component for the formation of functional NMDA receptors [18], and is widely distributed in the central nervous system [14].

It has been demonstrated that glutamate significantly increases in the spinal dorsal horn following peripheral inflammation and hyperalgesia [23], and intrathecal (i.t.) administration of glutamate agonist NMDA elicits pain behavior [16]. I.t. administration of dizocilpine maleate (MK-801), a non-competitive NMDA receptor antagonist, attenuates the hyperalgesia induced by peripheral inflammation [9,20,26,29]. Consistently, electrophysiological study has demonstrated that MK-801 inhibits the noxious heat-evoked response of dorsal horn nociceptive neurons, and reduces and prevents the expansion of the mechanical receptive fields of dorsal horn nociceptive neurons in rats following complete Freund's adjuvant (CFA)-induced peripheral inflammation [20]. These facts indicate that NMDA receptors are exaggeratedly activated during peripheral inflammation, contributing to the development of hyperalgesia and prolonged at the spinal level.

The proto-oncogene c-*fos* is rapidly and transiently expressed in response to noxious inputs in the central nervous system, including the spinal cord, and the immunocytochemical identification of c-Fos protein has been used as an indicator of activation of the nociceptive neurons at the spinal level [10]. Immunocytochemical studies have also demonstrated that MK-801 and 3-[($\pm$)-2-carboxypiperazin-4-yl]-propyl-1-phosphonic acid (CPP) can inhibit c-Fos expression induced by peripheral noxious stimulation in spinal cord [13], respectively. The aim of the present study is to demonstrate the co-localization of c-Fos and NMDA receptors in spinal neurons by using double immunostaining with both c-Fos antibody and NMDA

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NR1 (D2 Ab) antibody recognizing alternatively spliced carboxyl-terminals (lacking the C2 cassette) [11] in a rat model of chronic inflammation [24].

The combined detection of NMDA NR1 and c-Fos proteins was performed on four adult male Sprague–Dawley rats (250–300 g). A volume of 0.2 ml of CFA (*Mycobacterium tuberculosis*, Sigma), suspended in an oil/saline (1:1) emulsion, was injected subcutaneously into the plantar surface of unilateral hindpaw. This injection induced unilateral inflammation and behavioral hyperalgesia at the injected-hindpaw. At day 1 after CFA injection, when behavioral hyperalgesia peaked, the rats were deeply anaesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused through the left ventricle with 100 ml saline followed by 400 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The lumbar enlargement was removed and kept in the same fixative for 4 h, and then cryoprotected overnight in 30% sucrose in 0.1 M PB. The lumbar spinal cord was cut into transversal sections at 30 μm on a cryostat and collected in 0.01 M phosphate-buffered saline (PBS, pH 7.4) for immunocytochemical analysis. In order to abolish the endogenous peroxidase, the sections were treated with 0.1% NaN_3–0.3% hydrogen peroxide for 10 min [15]. Immunostaining was performed for NMDA NR1 first and then for c-Fos by the avidin–biotin–peroxidase (ABC) method at the same sections. Briefly, the sections were incubated in 10% normal goat serum with 0.3% Triton X-100 in PBS for 1 h and then incubated overnight in primary antiserum raised against carboxyl-terminal of NMDA NR1 (1:1000), and thirdly, incubated for 1 h in biotinylated goat anti-rabbit IgG (Vector, 1:400) and in ABC (Vector, 1:200) solution, respectively. Finally, the sections were developed in 0.02% diaminobenzidine (DAB) with 0.015% hydrogen peroxide in PBS, which resulted in reddish brown stain. The primary and secondary antibodies and the ABC were diluted in 2% normal goat serum with 0.3% Triton X-100 in PBS. Tissue sections were washed three times for 10 min each time in PBS between antibody incubations. All the incubations were carried out at room temperature. In order to abolish the peroxidase remained in the NMDA NR1 antibody–antigen complex in the tissue sections after reacting with DAB and hydrogen peroxide, the sections were treated with 0.1% NaN_3–0.3% H_2O_2 for 10 min again. The immunostaining for c-Fos was identical to the above methods with the exception that the primary antibody was rabbit anti-c-Fos (Oncogene, 1:20,000) and the peroxidase was reacted in 0.02% DAB with 0.015% H_2O_2 and 0.02% nickel chloride, which yielded a black stain. The sections were mounted on gelatin-coated slides, dehydrated in graded ethanol, cleared in xylene and coverslipped with DPX. In another two control rats, the animals were injected with saline into the hindpaws and treated the same way as above. The specificity of the primary NMDA NR1 antiserum has been reported previously [11]. In control for the immunocytochemical staining, when some sections were processed for immunocytochemistry described above with omitting the primary NMDA NR1 or c-Fos antibodies, no NMDA NR1 or c-Fos immunoreactive profiles were found. However, we acknowledge that the cross-reactivity of the c-Fos antibody with the proteins in the Fos family and any other unknown structurally related peptides or substances present in the tissue sections cannot be excluded and, therefore, the term c-Fos-like immunoreactivity (c-Fos-LI) is a more appropriate interpretation for Fos protein immunostaining. Similarly, the term NMDA NR1-LI was used too.

The stained sections were analyzed under an Olympus BH2 microscope for the distribution of NMDA NR1-LI neurons and of c-Fos-LI neurons within laminae I–X. Ten sections with the greatest number of c-Fos-LI neurons were selected from each animal for cell counting. The number of NMDA NR1-LI labeled and c-Fos-LI labeled as well as double labeled (NMDA NR1-LI/c-Fos-LI) neurons were counted mainly for laminae I–II and V of each selected section and averaged separately for sections of each rat, and then for the group.

In the present study, the reddish brown reaction product for NMDA NR1, with a specific antibody recognizing carboxyl-terminal region, is localized in cytoplasm and dendrites and does not enter the nucleus, which is consistent with the three transmembrane segment model of NMDA NR1 that the carboxyl-terminal of NMDA NR1 resides on the cytoplasmic side [18]. The dark reaction product associated with c-Fos is present in the nucleus. Thus, it is reliable to distinguish the single labeled NMDA NR1-LI and c-Fos-LI as well as double-labeled NMDA NR1-LI/c-Fos-LI neurons in the same sections.

The distribution of NMDA NR1-LI neurons in lumbar spinal cord of rat with unilateral CFA injection was illustrated in Fig. 1, in which there was no difference in the distribution of these neurons between two sides of the dorsal horn, and so was as compared to that in control rats (data not shown). Densely NMDA NR1 staining was found throughout the spinal laminae I–X, although some were lightly stained (e.g., in the lamina IX) than in others. Most of these neurons in lamina V were large multipolar in shape with dendrites being stained clearly, while most of them in superficial laminae were small to medium round and ellipse neurons. Under high magnification (40 × objective lens), NMDA NR-LI terminals can be identified in superficial laminae of the spinal dorsal horn (data not shown). c-Fos-LI nuclear profiles were mainly localized in the side ipsilateral to the CFA injection, and were also densely located in lamina V and laminae I–II, especially in the medial two thirds of the superficial dorsal horn (Fig. 2). Table 1 summarized that the cell countings on NMDA NR-LI labeled, c-Fos-LI labeled, and double labeled neurons in laminae I–II and lamina V in the side ipsilateral to the CFA injection. It was calculated that about 25% and 55% of c-Fos-LI nuclear profiles were founded in all NMDA NR1-LI neurons in superficial laminae and lamina

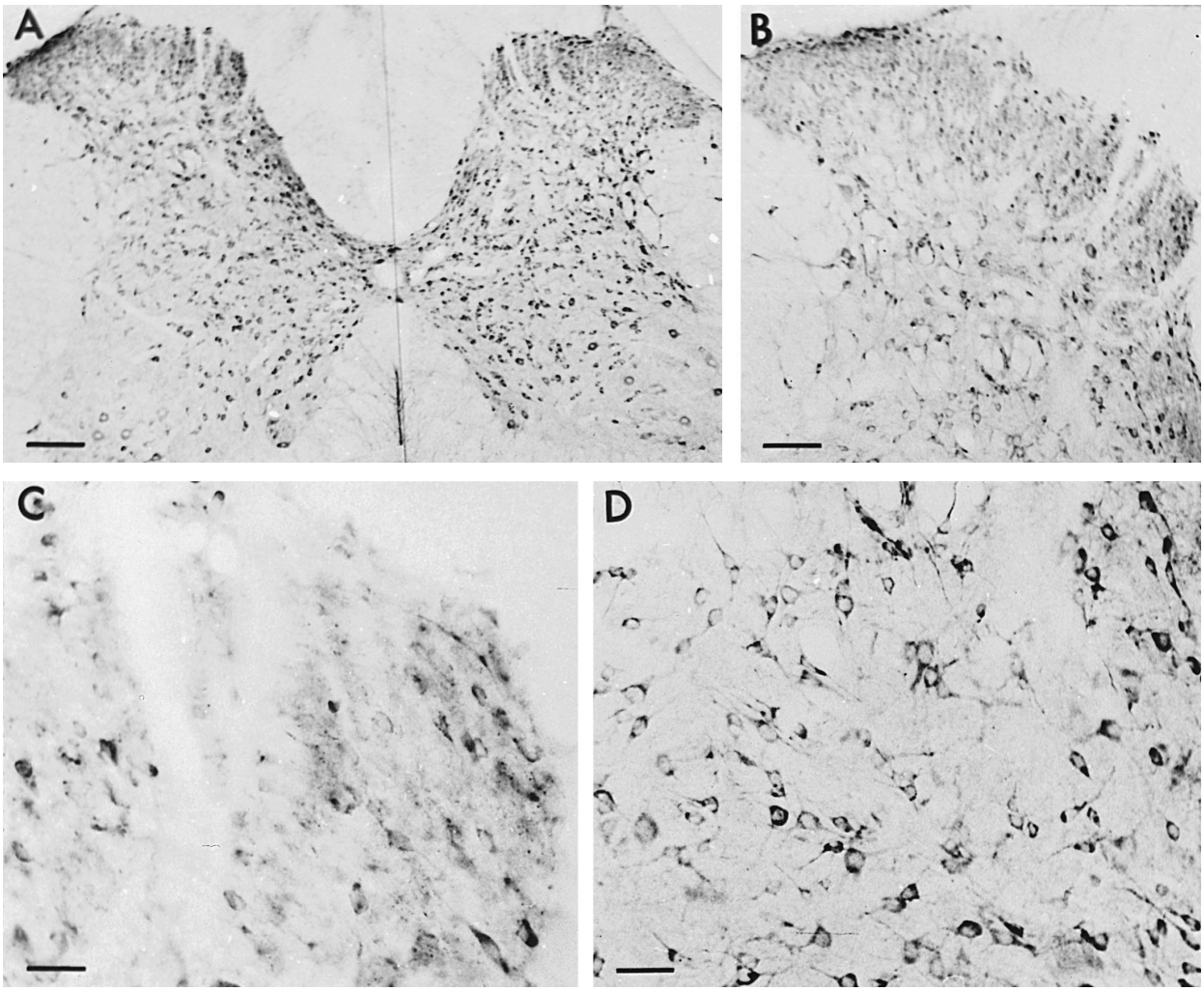

Fig. 1. Representative photomicrographs showing the distribution of NMDA NR1 immunoreactive neurons in lumbar spinal cord of rat with unilateral CFA-injection. NMDA NR1 is widespread throughout the spinal cord (A). Higher magnification of dorsal horn, superficial laminae, and lamina V is shown in (B), (C), and (D), respectively. Bars = 200 μm, 100 μm, 25 μm, and 50 μm in (A–D), respectively.

V, respectively (Table 1, 4th column), while about 4% and 11% of NMDA NR1-LI neurons showed c-Fos-LI in their nuclei in superficial laminae and lamina V (5th column), respectively. No NMDA NR1-LI/c-Fos-LI double-labeled cells were observed in laminae III–IV. Contralateral to the CFA injection, there were a few c-Fos-LI neurons, which were not analyzed further. In control rats, the c-Fos-LI profiles were only rarely observed in the dorsal horn (data not shown).

The present study has demonstrated that the NMDA receptor subunit NMDA NR1-LI peptide (lacking the C2 cassette) is widespread throughout the spinal cord neurons, which is consistent with previous in situ hybridization analysis showing that the NMDA NR1 mRNA distributes evenly in spinal cord gray matter [8]. The main finding of the present study is that 25% of c-Fos-LI neurons in the superficial laminae and 55% of c-Fos-LI neurons in lamina V are NMDA NR1-LI neurons. Since NMDA NR1 subunit is a key component of NMDR1–NMDAR2 heteromeric receptors and is believed to be the composition of native NMDA receptors [18], this fact implies that these double-labeled neurons are activated by the presynaptic release of glutamate that acts on the NMDA receptors of these neurons. If c-Fos expression is considered as an indicator of neurons that have been excited by the nociceptive inputs either directly or indirectly, a corollary should be achieved that about one-fourth of noxiously-activated neurons in laminae I–II and more than one-half of these neurons in lamina V receive glutaminergic terminations.

The question that needs to be answered is what implies the larger proportion, and also the larger total number, of double-labeled neurons in lamina V than that in superficial laminae. It has been generally accepted that NMDA receptor and non-NMDA receptors, such as AMPA receptor, are involved in the sensory transmission of both noxious and non-noxious stimuli, while different types of these receptors may play different roles in spinal nociception [1,17]. NMDA receptor antagonists have been documented to

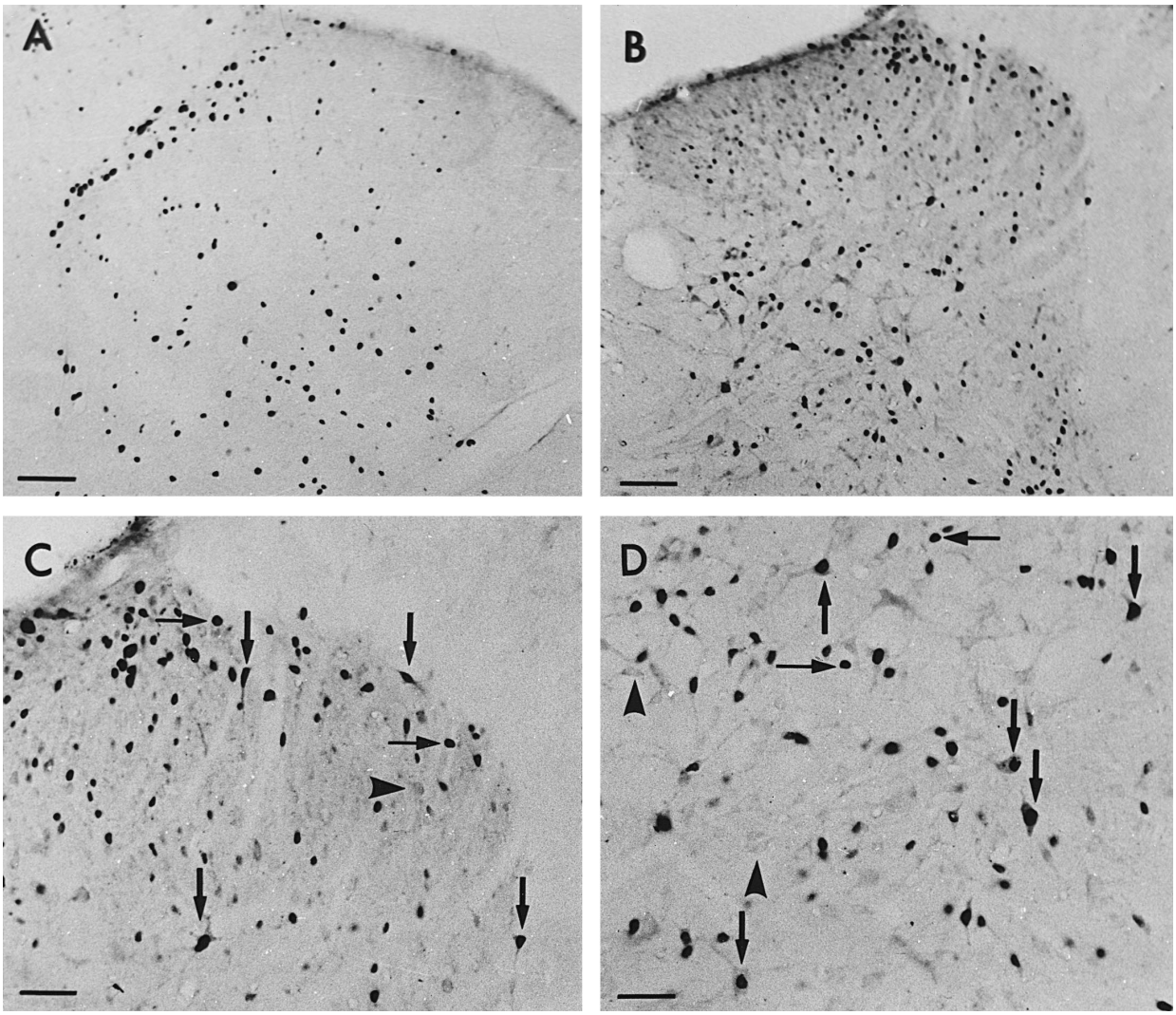

Fig. 2. Representative photomicrographs showing the distribution of c-Fos immunoreactive neurons (A) induced by ipsilateral inflammatory noxious stimuli, and the co-localization of immunoreactive NMDA NR1 and c-Fos (B). Higher magnification of superficial laminae and lamina V of panel (B) is shown in (C) and (D), respectively. Note that the immunostaining product for NMDA NR1, with a specific antibody recognizing carboxyl-terminal region, is localized in cytoplasm and dendrites, while immunostaining product associated with c-Fos is present in the nucleus. Vertical arrows indicate doubled labeled NMDA NR1-LI/c-Fos-LI neurons; horizontal arrows indicate single labeled c-Fos-LI neurons; arrowheads indicate single labeled NMDA NR1-LI neurons. Bars = 100 μm in (A–B) and 50 μm in (C–D).

inhibit the c-fiber-induced wind-up [6], the expansion of receptor fields after peripheral inflammation [20], and thermal hyperalgesia induced by carrageenan into joint [21] or by sciatic ligation [28]. Thus, NMDA receptors are preferentially involved in activity-dependent changes of excitability. On the other hand, nociceptive reflexes and single unit discharges of dorsal horn neurons appear to be mediated mainly through activation of AMPA receptors [5]. Taken together, it is proposed from the present experiments that lamina V is more importantly involved in the processes of wind-up and hyperalgesic state in chronic inflammation as compared to the superficial laminae. Previous studies have demonstrated that most of the nociceptive neurons in lamina V are predominantly the wide dynamic range cells and have a larger degree of spatial and/or modality convergence (reviewed in Refs. [2,27]). This may provide lamina V a possibility capable of doing easily in synaptic plasticity. Our proposal is supported by Chizh et al. [4] in a recent report showing that ketamine reduces the nociceptive responses of the majority of the

Table 1
Number (Mean ± S.E.) of c-Fos, NMDA NR1 and NMDA NR1/c-Fos immunoreactive neurons per section in the ipsilateral lumber dorsal horn 1 day after CFA injection

	c-Fos (1)	NMDA NR1 (2)	NMDA NR1/c-Fos (3)	(3)/(1)	(3)/(2)
I–II	45.2 ± 8.8	274.5 ± 35.4	11.6 ± 4.9	25.6%	4.2%
V	50.2 ± 9.7	258.3 ± 29.7	27.6 ± 3.4	54.9%	10.7%

The number is from 40 sections of four rats.

cells in deep dorsal horn and it also reduces the wind-up of the responses to repetitive electrical stimulation.

It should be mentioned that only small proportions of NMDA NR1-LI neurons in laminae I–II and lamina V, i.e., 4.2% and 10.7%, show c-Fos-LI labeling. This means that even in these regions most of the NMDA receptor-bearing neurons might not be involved in nociceptive processing and might play roles in other sensory functions.

Although glutamate-like immunoreactivity has been localized to primary afferent terminals [3] within the dorsal horn of the spinal cord, the decrease of glutamate level in the dorsolateral funiculus has been reported after rostral transection of the feline spinal cord [22]. Thus, the possibility that NMDA receptors in the dorsal horn may react to the glutamate released from terminals other than that from the primary afferent ones could not be excluded.

In conclusion, the present study demonstrates that more than one half of the c-Fos-LI neurons in lamina V possess NMDA receptors and this lamina may play more important roles in the activity-dependent changes of excitability during chronic peripheral nociception.

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